Pharmacologic Modulation of Picryl Chloride-Induced Contact Dermatitis in the Mouse

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A biphasic response of ear swelling was observed 2 h and 24 h after application of the antigen to picryl chloride-sensitized Balb/c mice. A platelet-activating factor (PAF) antagonist, BN 52063, or the anti-inflammatory drug, betamethasone, applied topically or injected subcutaneously, inhibited in a dose-dependent fashion the antigen-induced increase in ear thickness observed after 24 h. In addition, BN 52063 and betamethasone presented a synergistic effect when administered in vivo simultaneously and subcutaneously.

Indomethacin administered subcutaneously at the time of the antigen challenge significantly potentiated the early swelling phase and inhibited the late one. In contrast, the inhibitors of histamine and serotonin, ketotifen and methysergide, respectively, modulated mostly the early, and to a lower extent the late phase when administered at the time of antigen challenge. In contrast, none of these drugs inhibited the late phase reaction when administered 4 h after the antigen. A significant eosinophil and mononuclear-cell ear infiltrate was observed following topical application of the antigen, a phenomenon that was markedly reduced by either BN 52063 or betamethasone.

These results demonstrate the effectiveness of PAF antagonists, either alone or in association with glucocorticosteroids, in experimental CD, the modulation of the infiltration of eosinophils and mononuclear cells possibly explaining part of the inhibitory action of these drugs. *J Invest Dermatol 97:101–105, 1991*

Contact dermatitis (CD) is a complex skin disorder implicating various immunocompetent cells and mediators [1,2]. Besides lymphocytes, cell types including mononuclear phagocytes, mast cells, polymorphonuclear eosinophils, and neutrophils are required for the development of CD (reviewed in [1] and [3]). During the early phase following antigen challenge, these cell types are recruited upon the local generation of chemotactic factors produced by sensitized skin cells, mostly mast cells [4]. In addition, some of these mast cell-derived mediators, such as histamine and serotonin, induce a vasodilation and a vasopermeation of blood vessels around the site of the antigen challenge. Among the factors that alter skin homeostasis, and besides the preformed mediators, histamine and serotonin, newly formed ones like leukotrienes, prostaglandins, and platelet-activating factor (PAF) are also produced [5].

The phospholipid mediator PAF is of particular interest because of its various biologic and immunologic properties (reviewed in [6]). Indeed, this compound is generated by various cell types such as platelets, mast cells, endothelial cells, monocytes/macrophages, polymorphonuclear neutrophils, and eosinophils (reviewed in [6]). Interestingly, the majority of these cell types have been shown to be involved in CD. On the basis of these data, the possible implication of PAF in the specific and non-specific immunologic processes implicated in the development of allergic skin reaction was investigated using BN 52063, a mixture of ginkgolides containing BN 52020, BN 52021, and BN 52022 [7], as an antagonist. In addition, the pharmacologic effect of BN 52063, when given in association with the well-known glucocorticosteroid betamethasone, on the antigen-induced CD was investigated. Finally, the effect of the anti-serotonin drug methysergide, the anti-histamine ketotifen, and the cyclooxygenase blocker indomethacin on the antigen-induced CD was also analyzed.

MATERIALS AND METHODS

Animals Imbred male Balb/c mice (8–10 weeks old) obtained from Charles River (St-Aubin-les-Elbeuf, France) were used throughout the experiments. They were housed in our animal care facilities, fed standard mouse pellets, and given water ad libitum.

Sensitization Procedure The animals were sensitized by application on the abdomen of 0.15 ml of 7% picryl chloride (PiCl; BDH Laboratories, GFR) in an olive oil/acetone solution (1:9, v/v), as previously described [3]. This procedure was carried out under pentobarbital (45 mg/kg, intraperitoneal [IP]) anesthesia.

Administration of the Drugs BN 52063 (a standardized mixture containing the three ginkgolide compounds BN 52020, BN 52021, and BN 52022 in a weight ratio of 2:2:1, reviewed in [7]) was administered by two different routes: 1) topical application of 50 μl of a 2% cream, 1, 3, and 6 h after the antigen challenge, and 2) subcutaneous (SC) injection, 1 h before or 4 h after antigen challenge. In this latter case, a glycerol/0.15 M NaCl in water (1:1, v/v) solution was used as the vehicle. Betamethasone-17-valerate was used as a reference drug and was administered either topically as a 0.1% cream (Betneval, Glaxo Laboratories, Paris, France), 1 h

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Abbreviations:
CD: contact dermatitis
PAF: platelet-activating factor

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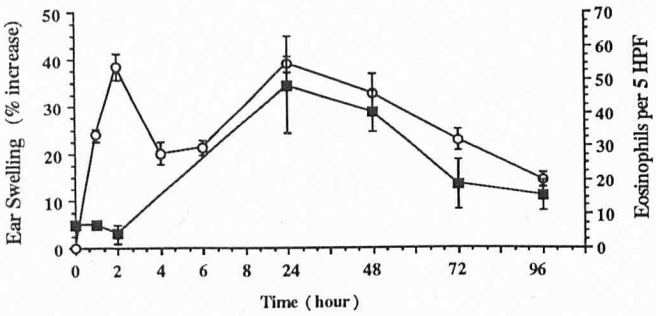

Figure 1. Kinetics of development of CD in the sensitized mouse. After various time intervals following antigen challenge, the alterations of ear thickness (○) and infiltration of eosinophils (■) were assessed, as described in *Materials and Methods*. The results are expressed as numbers of eosinophils per five high-power fields (HPF). Values are mean ± SD of the ones obtained in at least five animals.

after challenge, or subcutaneously (Celestene chronodose, Unicet Laboratories, Levallois-Perret, France), 1 h before or 4 h after challenge. In the studies where the effect of the association of betamethasone and BN 52063 was investigated, the drugs were injected SC, 1 h before antigen challenge and in separate regions of the back of the mice. Methysergide (Desernil, Sandoz Laboratories, Paris, France) was injected twice IP at the dose of 2.5 mg/kg, 18 h and 1 h before antigen challenge. In some experiments, methysergide was injected IP at the dose of 5 mg/kg, 4 h after the challenge with the antigen. Ketotifen (Zaditen, Sandoz, Paris, France) or indomethacin (Indocid, Merck, Sharp and Dohme, Paris, France) were injected IP and SC, respectively, at the dose of 5 mg/kg, either immediately prior to or 4 h after the challenge with the antigen. No interference of the various solvents of the drugs with the increase in ear thickness observed at various time intervals following antigen challenge was noted (data not shown).

Assessment of Ear Swelling Seven days after the sensitization of the animals, 50 μl of a 1% PiCl solution in olive oil/acetone was applied on each side of the right ear. In some experiments, the unchallenged left ear was treated with the drug or the vehicle alone for the evaluation of a possible irritant effect. Ear swelling was assessed after defined time intervals including the peak of the late response, i.e., 24 h after antigen challenge. The mice were anesthetized and the ear thickness was measured with a spring-loaded micrometer (Garnier, Boulogne, France). Every measurement was made in duplicate and the average of the two results was used to calculate a percentage of increase according to the following formula:

$$\frac{\text{thickness of the ear after challenge} - \text{thickness before challenge}}{\text{thickness of the ear before challenge}}.$$

Histologic Techniques Ear biopsies from treated and untreated mice were collected prior to and after defined time intervals following antigen challenge or vehicle administration. The specimens were fixed in buffered 10% formalin and then embedded in paraffin after dehydration in a series of solutions containing increasing concentrations of ethanol. Sections of 5 μm thickness were prepared and stained with LUNA specific for eosinophils and by the May Grünwald-Giemsa method for the determination of mononuclear cells [8]. Cells were counted in the corium between the epidermis and the cartilage. The counts were performed in five high-power fields arbitrarily chosen under a magnification ×400 and in a blind fashion.

RESULTS

Effect of Indomethacin, BN 52063, Betamethasone, and Methysergide on Antigen-Induced CD Application of the antigen on the right ear of sensitized mouse induced a biphasic increase in ear thickness (Fig 1). A first increase of ear swelling was noted as soon as 1 h after challenge and reached a maximum by 2 h. Then, a decrease in ear thickness was observed after 4–6 h that was followed by a maximum of increase after 24 h. The ear thickness progressively declined for up to 96 h (Fig 1).

In these experiments, the infiltration of eosinophils in the ear was also determined. The number of these cells started to increase at least 2 h after challenge with the antigen and up to a maximum reached after 24 h. The number of eosinophils progressively declined to reach values slightly higher than control ones after 96 h (Fig 1). Interestingly, no alteration in the number of eosinophils was noted during the first 2 h, i.e., the time period where the initial increase in ear thickness was observed. No alteration in the number of eosinophils in the ear was noted following challenge with any of the solvents used alone in the present study (data not shown). The number of mononuclear cells was also increased upon challenge of the animals with the antigen. Indeed, a net infiltration of 1192.0 ± 112.9 cells/five high-power fields was counted (n = 8).

In preliminary experiments, it was determined that three topical applications of BN 52063 (2% cream) 1, 3, and 6 h after antigen challenge induced optimal reduction of the antigen-induced CD. Thus, when a total dose of BN 52063 corresponding to 0.6 mg was topically applied, a highly significant reduction of the antigen-induced increase in ear thickness was observed, 24 h after the challenge. Indeed, the percentage of increase in ear thickness was 11.2 ± 0.4% (mean ± SD of 8 animals) and 46.3 ± 2.7% in BN 52063-treated and untreated animals, respectively ($p < 0.001$, t test). No effect of the cream vehicle of BN 52063 on the antigen-induced increase in ear thickness was noted (data not shown). In these experiments, betamethasone was more potent than BN 52063 because a single topical application of 0.01 mg of this drug almost totally inhibited CD. Moreover, a decrease in the thickness of the ear challenged with the antigen was observed following treatment with betamethasone, a result in keeping with the vasoconstrictor property of this drug [9]. In these experiments, no irritant effect of either BN 52063 or its vehicule was noted (data not shown).

In a next series of experiments, to avoid a possible non-specific interference of the drugs with the development of antigen-induced CD, the animals were injected with either indomethacin (SC), BN 52063 (SC), betamethasone (SC), methysergide (IP), or ketotifen (IP) at distance of the site of antigen challenge. All drugs were administered 1 h before antigen challenge with the exception of methysergide, which was given twice 18 h and 1 h prior to initiation of CD. The results presented in Fig 2 demonstrated that only the antagonists of serotonin, methysergide, histamine, and ketotifen were able to modulate the initial phase of CD observed 2 h after the challenge. In contrast, treatment of the animals with indomethacin markedly and significantly enhanced the increase in ear thickness observed as soon as 1 h after challenge and for up to 4 h. This drug, as well as BN 52063 and betamethasone, however, reduced the late phase of CD noted after 24 h. A significant inhibition of the late increase in antigen-induced ear thickness by methysergide and ketotifen was also noted.

The dose-dependent effect of a single SC administration of BN 52063, at doses ranging from 1.25–40 mg/kg, on the late ear swelling evoked 24 h after challenge with the antigen was investigated (Fig 3). In this series of experiments, the injection of BN 52063 was performed 4 h after challenge, i.e., at the beginning of the late phase. As presented in Fig 3, BN 52063 inhibited the antigen-induced ear swelling in a dose-dependent manner. Moderate but significant inhibition of antigen-induced CD was noted with the dose of 1.25 mg/kg BN 52063 and maximal effect was produced at 40 mg/kg. Similar experiments were also conducted with betamethasone injected 4 h after the challenge with the antigen and at doses ranging from 0.5–8 mg/kg. As presented in Fig 4, this drug inhibited the antigen-induced ear swelling in a dose-dependent manner and with a maximal effect at the dose of 8 mg/kg. The fact that under those experimental conditions both BN 52063 and betamethasone reduced the late CD in mouse indicates that their action is not related to the inhibition of an early event (occurring during the first 4 h) of the allergic skin reaction.

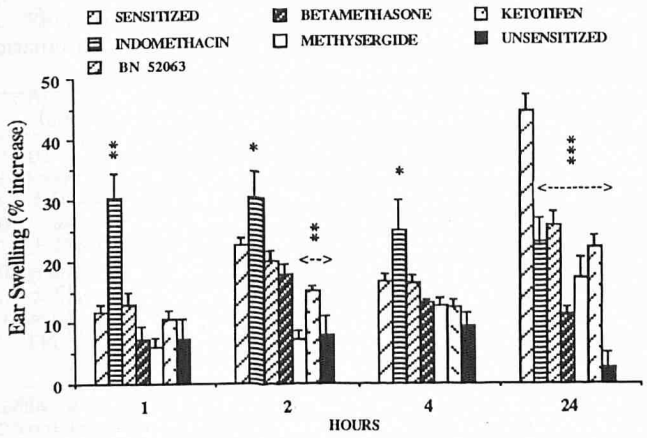

Figure 2. Inhibition of antigen-induced CD in the mouse after administration of various drugs. Indomethacin, BN 52063, and betamethasone were administered SC 1 h before the antigen challenge (at 5, 40, and 8 mg/kg, respectively). Methysergide was injected IP 18 h and 1 h before the challenge (2.5 mg/kg in each case). Ketotifen (5 mg/kg) was also injected IP 1 h before the challenge. *Bar,* mean ± SD of the values obtained in at least five animals. For each time point, the values from the various groups of treated animals were assessed for statistical difference with respect to the data obtained in the group of sensitized and challenged mice. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

A negligible effect of methysergide and ketotifen administered 4 h after challenge on the late increase in ear thickness was noted (Fig 5). In contrast, indomethacin administered 4 h after the antigen significantly inhibited the increase in ear thickness observed after 24 h (Fig 5).

Effect of Treatment with BN 52063 and Betamethasone on the Antigen-Induced Cell Infiltration in the Ear Histologic examination of ear sections demonstrated significant differences between untreated sensitized and challenged animals and those topically receiving BN 52063 (Fig 6). A marked decrease in the number of eosinophils in the ear was noted in the animals treated with BN 52063 (a dose corresponding to 0.6 mg), 1, 3, and 6 h after challenge with the antigen (Fig 6). Betamethasone was even more active when compared to BN 52063 because the infiltration of eosinophils was totally inhibited. No effect of the cream vehicle of BN 52063 on the antigen-induced eosinophil infiltration was noted (data not shown).

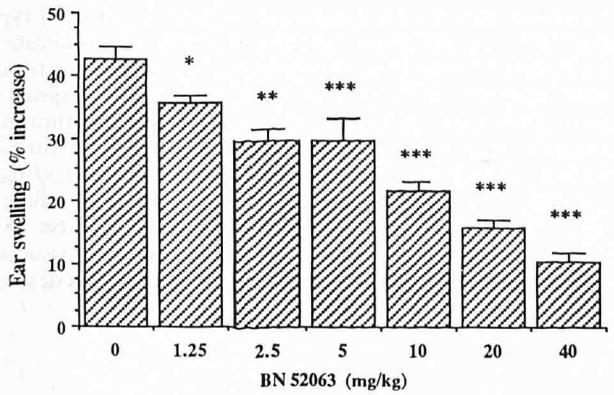

Figure 3. Inhibition of antigen-induced CD in the mouse after SC administration of BN 52063 at various doses 4 h after challenge. The experiments were conducted as described in *Materials and Methods. Bar,* mean ± SD of the values obtained in at least 10 animals. As a control, animals were injected with the vehicle alone *(bar 0)*. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

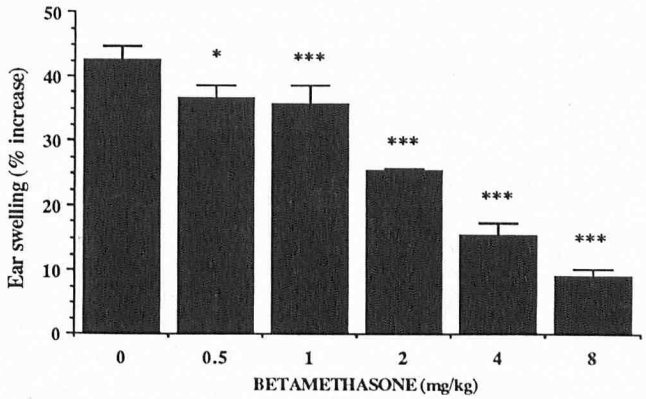

Figure 4. Inhibition of antigen-induced CD in the mouse after SC administration of betamethasone at various doses 4 h after antigen challenge. The experiments were conducted as described in *Materials and Methods. Bar,* mean ± SD of the values obtained in at least eight animals 24 h after antigen challenge. As controls, animals were injected with the vehicle alone *(bar 0)*. $*p < 0.05$ and $***p < 0.001$.

In parallel with the number of eosinophils, that of mononuclear cells was also decreased. An average of 298.7 ± 12.7, 573.0 ± 44.9, and 1192.0 ± 112.9 cells was measured in five high-power fields, 24 h following challenge with the antigen in animals treated with betamethasone, BN 52063, or vehicle, respectively ($p < 0.01$, $n = 8$).

Effect of Treatment with the Association of BN 52063 and Betamethasone on the Antigen-Induced CD The fact that betamethasone and BN 52063 selectively decreased the late phase of CD and affected cell infiltration led us to evaluate a possible additive or synergistic effect of their association in vivo. The data obtained with various combinations of dosages demonstrated that maximal inhibition was produced with a dose of 4 mg/kg betamethasone, given in association with 2.5 mg/kg BN 52063 (Table I). Increasing the dose of the PAF antagonist up to 10 mg/kg did not improve the effect of 2 mg/kg betamethasone in a significant fashion. Finally, as the effect of these drugs in combination was not significantly different from that obtained by simultaneous injections of both, it was concluded that their effect was rather additive than synergistic.

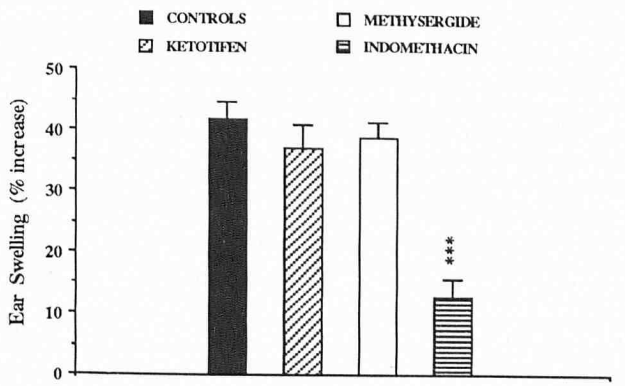

Figure 5. Effect of various drugs on antigen-induced CD observed after 24 h in the mouse. Ketotifen (5 mg/kg, IP), methysergide (5 mg/kg, IP), or indomethacin (5 mg/kg, SC) were injected 4 h after the antigen challenge. *Bar,* mean ± SD of the values obtained in at least five animals. $***p < 0.001$.

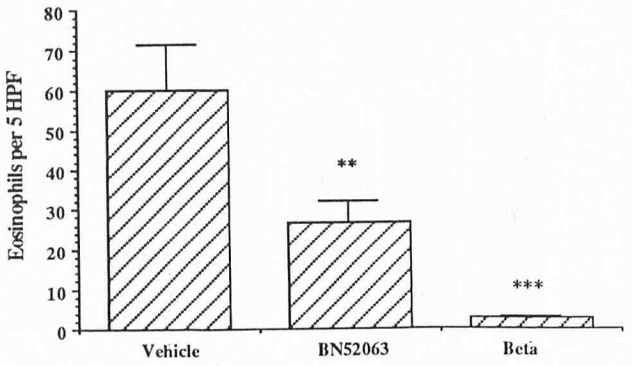

Figure 6. Inhibition of antigen-induced eosinophil infiltration observed in the mouse after triple topical application of either BN 52063 (1, 3, and 6 h prior to challenge) or betamethasone *(Beta)* or cream vehicle alone (1 h after challenge). The experiments were conducted as described in *Materials and Methods.* Ear biopsies were collected 24 h after antigen challenge. The eosinophil counts were performed in a double-blind fashion and on five high-power fields (HPF) arbitrarily chosen from the apex of the ear (magnification ×400). *Bar,* mean ± SD of the values obtained in at least eight animals.

Table I. Effect on Antigen-Induced Ear Swelling of Betamethasone and BN 52063, Either Alone or In Combination at Various Dosages[a]

Betamethasone (mg/kg)	Associated Dose of BN 52063 (mg/kg)		
	0	2.5	10
0	42.70 ± 2.07 (n = 18)	29.69 ± 1.96 (n = 12)	21.80 ± 1.43 (n = 10)
0.5	36.59 ± 2.0 (n = 12)	20.21 ± 1.57[b] (n = 12)[d]	14.81 ± 1.72[b] (n = 10)[c]
2	25.37 ± 0.23 (n = 15)	7.87 ± 1.81[b] (n = 7)[c]	6.47 ± 0.31 (n = 5)
4	15.47 ± 1.92 (n = 9)	2.74 ± 0.32 (n = 7)[c]	NT

[a] Both drugs were injected SC 1 h before antigen challenge and in two different locations. The results are expressed as percent of increase in ear thickness, measured 24 h after antigen challenge. For each combination of drugs, statistical analysis was performed with respect to the values obtained in animals treated with betamethasone ([b] $p < 0.001$) or BN 52063 ([c] $p < 0.05$, [d] $p < 0.01$, [e] $p < 0.001$) used alone and at the same dosage. NT, not tested.

DISCUSSION

The present results demonstrate that the PAF antagonist BN 52063 decreases the extent of allergic CD, suggesting that this mediator could play a role in this phenomenon. This effect of BN 52063 on the antigen-induced increase in ear thickness is maximal after a triple topical application of the drug. However, to exclude a possible non-specific action of BN 52063 on the ear swelling, experiments in which the drug was given SC at a distance from the site of antigen challenge were also conducted. In this case, a dose-dependent inhibition was observed, with a plateau effect at doses of BN 52063 higher than 20 mg/kg. This result demonstrates that the effect of BN 52063 is specific and most likely related to its PAF antagonistic activity.

In agreement with previous reports [9], a marked inhibition of antigen-induced CD was observed after a single topical application of betamethasone. As for BN 52063, betamethasone also inhibits CD in a dose-dependent manner when administered SC. In addition, our results show that these two drugs present a similar pattern of inhibition because they selectively modulate the late phase of CD. In contrast, methysergide and ketotifen modulate both the early vascular and the late inflammatory phase of CD. This latter effect of methysergide and antihistamine drugs has been already described by Askenase et al [4], who demonstrated the primary role of sensitized mast cells and vasoactive amines in the triggering of the skin allergic reaction. The fact that both methysergide and ketotifen failed to affect the late phase of CD when administered 4 h after antigen challenge indicates that their effects, depicted in Fig 2, are related to the inhibition of an early event following triggering of the reaction.

The similar pattern of inhibition obtained with BN 52063 and betamethasone when used separately led us to investigate the effect of their association in vivo and to demonstrate an additive pharmacologic action. Obviously, this effect is difficult to explain because the influence of corticoids on inflammatory responses in vivo is highly complex [10].

In our study, the inhibition of antigen-induced CD by BN 52063 and betamethasone was also associated with a 50% reduction in the number of eosinophils and mononuclear cells infiltrating the site of antigen challenge. Interestingly, an inhibitory effect of glucocorticoids on the in vitro chemotaxis of eosinophils has been previously described [11]. However, the relationship between the development of CD and eosinophil and mononuclear cell infiltration is presently unknown, although the possibility that the former cell type could be of primary importance in IgE-dependent allergic late-phase skin reaction in humans has been raised recently [12].

Although the implication of T lymphocytes in the development of cutaneous allergic reactions has been extensively studied in experiments involving adoptive transfer of this cell type, the relationship between IgE production (leading to mast cell sensitization and further degranulation upon antigen challenge) and the release of T cell–derived factors that secondarily induce mast cell degranulation is still unclear. Given the fact that active sensitization of the animals was performed in the present study, the possibility that both mechanisms are operational remains. Therefore, it is very difficult to conclude whether the presently reported pharmacologic modulation of the increase in ear thickness is in relation to the inhibition of a purely T cell–dependent process of the late phase of an IgE- and mast cell–dependent type I hypersensitivity reaction, or both. In preliminary experiments, and as assessed by the passive cutaneous anaphylaxis reaction, the presence of circulating PiCl-specific IgE in the plasma of sensitized animals was investigated. Although no such presence of specific IgE could be demonstrated, this result does not rule out the possibility that IgE have indeed been produced in amounts sufficient to sensitize mast cells without entering the circulation. Further studies are obviously required to determine the relative contribution of the various cellular and humoral components initiated upon contact sensitization.

The present results indicate that PAF could play a role in the elicitation of CD, possibly by increasing vascular permeability and attracting inflammatory cell types such as eosinophils. Indeed, PAF is now recognized as a potent modulator of eosinophil functions and is one of the most potent chemotactic agents for this cell type in humans [13–16]. Although the bulk of these results indicate that PAF is a primary agent in the triggering of eosinophil functions, its modulating effect on CD may be related to other mechanisms such as the modulation of cytokine production or the amplification of various cell responses by other agonists [17,18]. Further, the possibility that PAF might affect cell types such as monocytes/macrophages and T cells, which also play a role in CD, is still open. Finally, from a clinical point of view, the additive effect of BN 52063 and betamethasone is of interest because their association may open a new therapeutic approach for the treatment of allergic skin diseases.

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